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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this study is to understand the role of the p16 growth control axis in androgen dependent proliferation of prostate cancer cells. The p16 axis contains two tumor suupressors (p16Ink4a and pRB), cyclin D-dependent kinases, and transcription factor E2F. We hypothesized that functions of the p16 axis can influence androgen-dependence of prostate cancer cells. To test this hypothesis, we proposed to use controlled expression techniques to determine whether disruption of p16 axis function can lead to androgen-independence in human androgen-dependent prostate cancer cells (LNCaP) and, on the other hand, whether restoration of p16 axis function can restore androgen-dependence in androgen-independent prostate cancer cells (DU-145). During the first year of this study, we have established conditions to establish LNCaP derivative cell lines with inducible expression of exogenously introduced proteins. For DU-145 cells we have found an essential role of pRB in maintaining androgen receptor expression. This finding may provide a missing link between androgen receptor signalling and the tumor suppression function of the p16 axis. We have established pRB inducible expression lines from DU-145 to determine the molecular mechanisms of this function of pRB.

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Introduction

The subject of this proposal is the role of the p16 growth control axis in androgen dependent proliferation of prostate cancer cells. The p16 axis contains two tumor suppressers (p16Ink4a and pRB), an important component of the cell cycle machinery (cyclin D-dependent kinases), and transcription factor E2F (regulators of gene expression). We hypothesized that functions of the p16 axis can influence androgen-dependence of prostate cancer cells. To test this hypothesis, we proposed to use controlled expression techniques to determine whether disruption of p16 axis function can lead to androgen-independence in human androgen-dependent prostate cancer cells and, on the other hand, whether restoration of p16 axis function can restore androgen-dependence in human androgen-independent prostate cancer cells. Through this study, the connection between androgen dependent growth of prostate cancer and functions of a central cell growth control pathway will be revealed.

Body

The two approved aims in this project are discussed separately below.

Aim 1. To determine whether deregulated expression of positive-acting cell cycle regulators can abrogate the dependence of LNCaP cells on steroid hormones in culture.

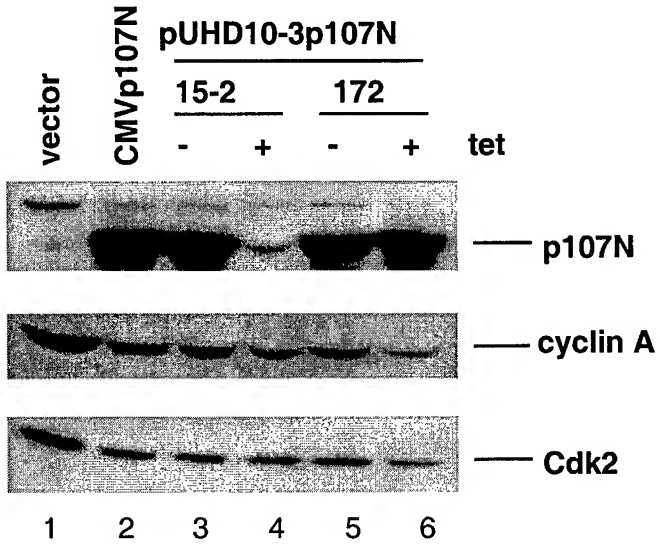

Figure 1. Tetracycline controlled expression in LNCaP prostate cancer cells.

LNCaP cells were transfected with the indicated plasmids using Fugene 6 (Boehringer Mannheim). In lane 1, cells were transfected with vector alone as a control. In lane 2, pCMVp107N is a constitutive expression vector expressing the p107N protein. In lanes 3 and 4, cells were transfected with the tetracycline responsive vector pUHD10-3p107N together with the tTA expression vector pUHD15-2 in the presence and absence of tetracycline as indicated. Lanes 5 and 6 were similarly transfected except the rtTA expressing vector pUHD172 was used. Total cell extracts were subjected to SDS gel electrophoresis and immunoblotted with the indicated antibodies. The expression of p107N is highly controlled by pUHD15-2, but not pUHD172. The levels of cyclin A and Cdk2 serve to indicate the loading of cell extracts in each lane.

The first objective of this aim is to establish LNCaP derivative cell lines that can express various cell cycle regulators in the p16 axis in a controlled manner. Since we are new to the prostate cancer field, we first learned how to culture LNCaP cells, and confirmed that they are androgen-dependent for proliferation in culture. We then optimized transfection protocols for these cells. Next, we tested two types of tetracycline inducible systems to determine the best protocol to establish inducible expression in LNCaP cells, as described below.

The tetracycline inducible expression technology was first reported in 1992 (2). In the first report, the transactivation activity of the tetR-VP16 fusion transactivator (tTA) was repressed by tetracycline. The activation of gene expression therefore is achieved by withdrawal of tetracycline in the media (tetracycline was present at all times after cells are transfected with the transactivator and the expression construct). In 1995, a mutant version of tetR was reported which was activated by tetracycline (rtTA) (3). With this system, the induction of gene expression is

achieved by adding tetracycline to the media.

We tested both inducible expression systems in LNCaP cells. In the experiment shown in Figure 1, we transfected LNCaP cells with the tetO based expression vector containing a fragment of the p107 gene (pUHD10-3p107N, p107N was used only as a marker protein) together with either tTA (pUHD15-2) or rtTA (pUHD172). For each transfection, we compared the expression of p107N in the presence and absence of tetracycline. The results clearly showed that LNCaP cells are much better suited for tTA mediated gene induction. With tTA, p107N expression is very low in the presence of tetracycline (OFF state, lane 4) and is induced to significant levels after tetracycline withdrawal (ON state, lane 3). In contrast, rtTA mediated induction showed unacceptable levels of leaky expression in the OFF state (absence of tetracycline, lane 5). Therefore, we have decided to use the tTA transactivator to establish inducible LNCaP cell lines. We transfected the tTA construct (pUHD15-2) with a neomycin drug resistance marker plasmid (pSVneo) into LNCaP cells and selected clones by resistance to neomycin (G418). These clones are currently being characterized to determine their ability to regulate the expression pUHD10-3p107N.

We expect to obtain tTA expressing LNCaP cell clones in the next one to two months. Once these clones are established, we will transfect them with tetO based expression constructs (pUHD10-3) containing various cell cycle regulators in the p16 axis, including cyclin D1, cyclin E, cyclin A, Cdk4, Cdk2, and E2F1 and E2F3 as originally planed.

<u>Aim 2. To determine whether correction of p16 axis function and androgen receptor expression can restore androgen dependence in DU-145 cells.</u>

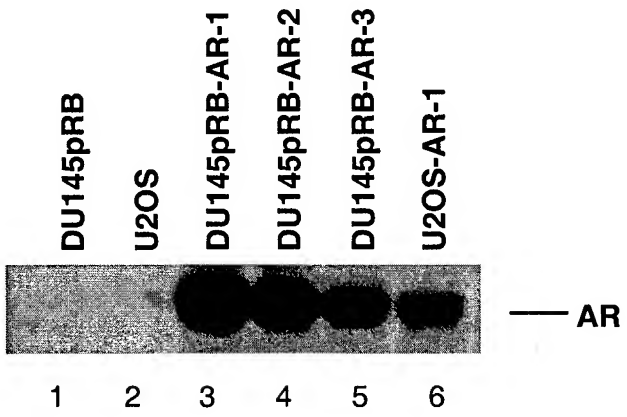

Figure 2. Restoration of androgen receptor expression in androgen receptor negative DU-145 prostate cancer cells.

DU1-145pRB cells were stably transfected with pCMV-AR and a puromycin resistent marker for selection. Clones of cells were isolated with cloning rings and expanded. Three (lanes 3, 4, and 5) out of six clones examined contain androgen receptor expression as determined by Western blotting with an anti-AR antibody (sc-815 from Santa Cruz Biotechnology). The position of AR is indicated. U2OS is a human ostosarcoma cell line with functional pRB. U2OS cells with constitutive expression of AR can also be established (lane 6).

DU-145 prostate cancer cells contain non-functional mutant pRB (1). It was reported that reexpression of wild type pRB in a constitutive expression vector in DU-145 cells could repress their tumorigenicity in nude mice but did not affect their proliferation in regular culture medium containing high concentrations of serum (1). To study the androgen dependence nature of DU-145 cells, it is first necessary to restore androgen receptor expression since they do not express androgen receptor (AR).

To restore AR expression in DU-145 cells, we transfected DU-145 cells with pCMV-AR (containing a neomycin resistant gene for drug selection of stable clones) and screened about fifty clones selected by neomycin resistance. None of the clones was able to express AR, suggesting that AR expression is not tolerated in DU-145 cells. In contrast, when we transfected a derivative of DU-145 cells that constitutively express wild type pRB (1), we obtained AR-expressing clones with high

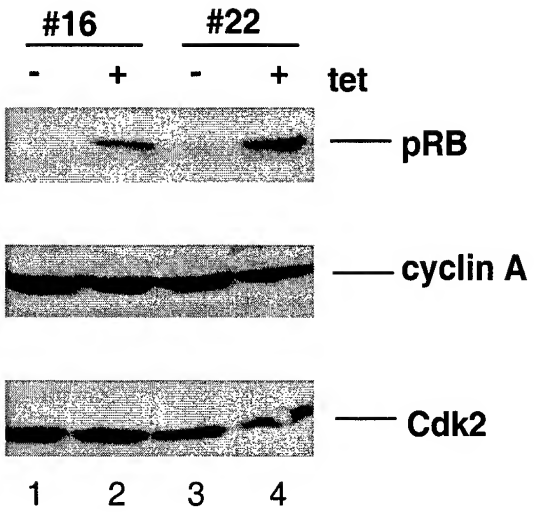

Figure 3. Generation of DU-145 derivative lines with inducible expression of pRB.

Two independently established lines are shown (#16 and #22). Tetracycline was added to the culture media for twenty four hours, as indicated. Total cell extracts were subjected to SDS gel electrophoresis and immunoblotted with the indicated antibodies. As shown, the expression of pRB is under control of tetracycline. The levels of cyclin A and Cdk2 serve to indicate the loading of cell extracts in each lane.

frequency (Figure 2 shows three clones, lanes 3, 4, and ,5 obtained from a screen of six clones). AR-expressing cell lines were also readily established in another tumor cell line with functional pRB (U2OS human osteosarcoma cell line, Figure 2 lane 6). These results suggest that pRB function is important for maintaining AR expression in prostate cancer cells. This finding may provide an important link between the p16 axis and the AR signaling pathway.

The attempt to establish inducible cell lines from DU-145 has been successful. We have now established DU-145 derived cell lines that inducibly express functional pRB. We used the rtTA tetracycline controlled expression system in this experiment. This system, although very leaky in LNCaP cells as shown above, does not show significant amount of leaky expression in DU-145 cells. Figure 3 demonstrates two independently obtained inducible clones (clone 16 and clone 22). Expression of wild type pRB in these two clones can be induced by the addition of tetracycline in the culture media.

With the establishment of pRB inducible DU-145 cells, we are transfecting AR vectors in the presence of pRB expression to allow stable expression and restoration of AR. We can then turn off pRB expression (mimicking pRB mutation during the course of prostate cancer progression) by withdrawal of tetracycline in the media to directly determine the effect of pRB on AR expression. Once the effects that we observed in DU-145 cells with constitutive expression of pRB are confirmed, our inducible cell lines will allow us to determine the underlying mechanisms of the pRB effects on androgen signaling.

Key Research Accomplishments

- Established conditions for the generation of LNCaP tetracycline controlled expression cell lines.
- Identified pRB as a potential regulator of AR expression in DU-145 cells.
- Established DU-145 cell lines that can express pRB in a controlled manner.

Reportable Outcomes

Not available.

Conclusions

The originally reported tetracycline inducible expression system is well suited for inducible expression in LNCaP cells.

One functional role of the tumor suppresser pRB in prostate cells may be to retain the expression of androgen receptor. DU-145 cells with inducible expression of pRB can be used to determine the mechanisms of pRB effects on AR expression.

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